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Comparative sequence data suggest that mammalian phosphofructokinase(PFK) has evolved from a procaryotic precursor by gene duplication, fusion, and mutation of previous catalytic sites into new regulatory ligand binding sites. We are using two approaches to examine this problem. We are attempting to duplicate these events by recombinant DNA technology. Using a synthetic oligonucleotide that matches the mammalian link peptide we are joining 2 E. coli PFK genes, analyzing the product, and mutagenizing the product. In the second approach, we are looking at two unique PFKs, a potato enzyme, and one from Propionibacteria to determine their evolutionary path. Despite great overall molecular differences, antibody suggest similarities among the various PFKs.

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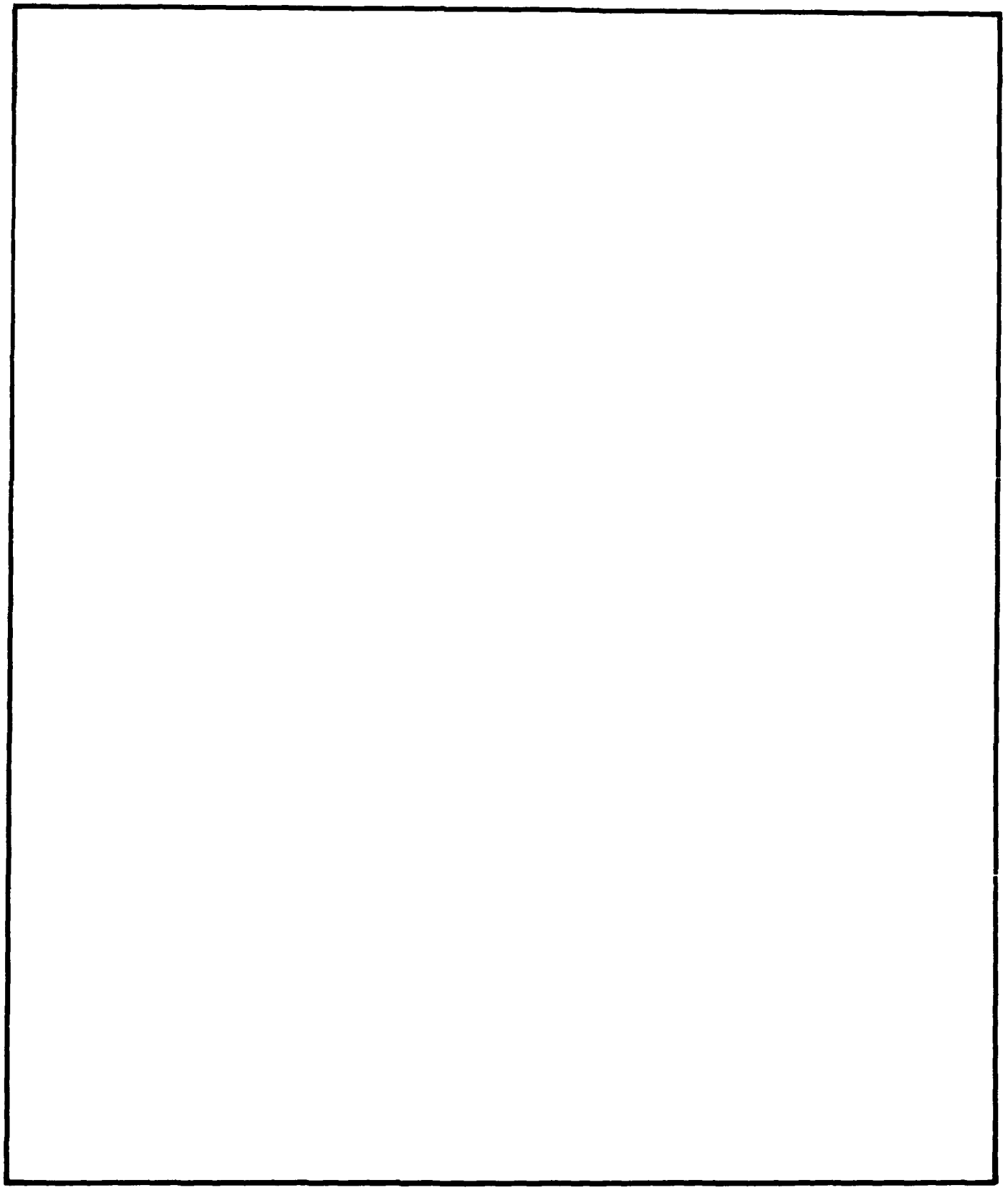

PROJECT TITLE: EVOLUTION OF PHOSPHOFRUCTOKINASE

Final Report for the period 8/1/86 - 7/31/89

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Project Goals:

The overall goal of this project is to establish the relationship between procaryotic and eucaryotic phosphofructokinase (PFK). Comparative sequence data suggest that eucaryotic PFK has evolved by gene duplication, fusion, and mutation of previous catalytic sites into new regulatory ligand binding sites. Two approaches have been used: one was to examine the structures of PFKs with widely divergent properties and the second was an attempt to use molecular biology to duplicate the fusion/mutation events. The *E. coli* pfk gene was recloned into a convenient plasmid. Using a synthetic oligonucleotide that matches the mammalian "link" region, we attempted to join two *E. coli* pfk genes. Utilizing site-directed mutagenesis, conversion of one of the two catalytic to a regulatory site was attempted.

RESULTS

Attempts to Fuse Genes:

The PFK gene along with a strong upstream promoter was removed from pLC16-4 by cleavage with SinI and HpaI. HindIII linkers were ligated to the fragment and the fragment was inserted into the HindIII site of pUCmp8. Very "sick" colonies were produced and the only successful M13 insertion involved extensive deletions from the 5' end of the gene was indicated by restriction maps. After numerous failures, we decided that a strong promoter present with the fragment were leading to a transcript that was somehow disturbing the host cell's normal transcription. The vector pKK232-8 has a promoter-less chloramphenicol transacetylase gene long with unique transcription terminators. We cloned into the HindIII site of this vector and generated an excellent PFK overproducer that grew in the presence of ampicillin and chloramphenicol. This plasmid has been named pSLJ-3. PFK was overproduced by approximately 10-fold when this vector was used to transform *E. coli* L#392. The HindIII linked PFK was circularized, restricted at the BGlII site and inserted into pSLJ-7, which was constructed by putting an additional BglII site into a BAmH1/HindIII site of pKK232-8. The new construct, pSLJ-2 serves as a vector for manipulations of the "linker" for insertion into the BglII site of pSLJ-3 to create the "duplex". Oligonucleotides to be used to assemble the "linker" region between two PFK genes were synthesized. In general, the strong promoter continued to plague attempts to create a duplex gene, and it was decided to develop an alternative approach by eliminating the promoter and cloning into more conventional vectors.

Removing promoter and other mutagenesis

A second area of investigation has been the production of site-directed mutants that will be useful in altering one of the two catalytic sites of our duplex. A mutation was made by the method of Mandecki, which involves introducing a single restriction site cleavage into the gene and transforming with the cut plasmid plus an oligonucleotide that bridges the cut and contains one or

more mismatches near the restriction site. We cleaved pSLJ3 with BglII and added a 65-mer that removed the restriction site and changed G174 to E. G174 is three residues from critical catalytic residue involving ATP and seems to be important for the folding of the pocket. The kinetic properties of the mutant enzyme are fairly similar to native enzyme except for a low Vmax. Further studies have shown that the low Vmax was apparent and that the actual reason for the low values was the instability of the enzyme. Heat denaturation studies demonstrated that the mutant was denatured rapidly at 40 to 42°C whereas the wild-type enzyme denatured in the range of 60 to 62°C.

pSLJ3 is an excellent overproducer of PFK, but we still had the problem of cloning into M13 which is useful for most of the techniques of mutagenesis. The approach that succeeded was a Bal digest of the HindIII linked PFK gene (actually from a single end at the polylinker region). This generated a promoter-less PFK gene that we are now putting into M13, pUC, and other vectors. This will facilitate all aspects of our project.

Other PFKs

We have looked at two unusual PFKs with regard to their relationship to the major PFK family. The PPi-dependent enzyme from *Propionibacteria* has been studied. The enzyme was digested with 2 different proteases, and peptides were isolated by HPLC. Approximately 200 residues of its 440-460 residues have been sequenced, and oligonucleotide probes have been synthesized. A genomic library has been generated and is now being probed.

In addition, a procedure developed for the purification of inorganic pyrophosphate; fructose-6-phosphate 1-phospho-transferase (PPi-PFK) from potato tubers. The enzyme has the structure $\alpha_4\beta_4$ with a subunit of 68kDa and a ß subunit of 60 kDa. The structural relationship of the enzyme to other PFKs and to fructose bisphosphatase was examined by immunoprecipitation and immunoblotting. Antibodies to the plant enzyme did not react with *E. coli* PFK. No cross-reaction was seen among the following enzymes or their antibodies: yeast fructose bisphosphatase; rabbit PFKs A,B, or the enzyme from brain; and the two subunits of the potato PPi-PFK. On the other hand, antibody to *E. coli* PFK-1 strongly cross-reacts with the 60 kDa polypeptide but not 68 kDa peptide.

The data support the idea that the plant PPi-PFK has evolved from the same procaryotic progenitor that led to the major ATP-dependent enzyme of *E. coli* and to the mammalian PF-1-Ks. The structural identity of the second subunit, α, and its role in the reaction remain to be established. Three short tryptic peptides of the 60 kDal and one of from the 68 kDal have been sequenced. Oligonucleotides are being synthesized on the basis of these sequences to probe a plant cDNA library.

In addition, we have recently partially purified ATP dependent PFK potato tubers. Using the Western blot technique, we have shown that antibody to the 60 kDal subunit of the PPi-PFK is capable of recognizing a subunit of the ATP dependent enzyme.

Inventions: None

Publications:

Yuan, X.-H., Kwiatkowska, D., and Kemp, R.G. (1988) Inorganic pyrophosphate: fructose-6-Phosphate 1-Phosphotransferase of the Potato Tuber is Related to the Major ATP-Dependent PFK of E. coli. Biochem.Biophys.Res.Commun. 154, 113-117.

Other manuscripts will be forthcoming.

Training:

X.H. Yuan (Postdoctoral Research Associate)
Sangwei Lu (Graduate Student, 4 months).